To the Editor:

In a recent issue of Clinical Chemistry Janssen et al. (1) reported the development of a new spectrophotometric assay to determine complex I activity in a mitochondrial fraction of human skin fibroblasts, which is based on measuring the reduction of 2,6-dichloroindophenol by electrons accepted from decylubiquinol. This is a potentially important finding because the determination of complex I in fibroblasts is difficult owing to the high activity of contaminating rotenone-insensitive NADH dehydrogenases (2). In the reported method complex I was assayed by measuring the total NADH oxidase activity during a 4-min period, after which rotenone was added to measure the rotenone-insensitive NADH oxidase activity. By subtraction of the reaction rates, the complex I activity was calculated. Because it is well known that the accumulation of rotenone on its binding site is not instantaneous (3, 4), we questioned if the complex I assay might be affected by the delay in the inhibitory effect of rotenone.

We investigated the relationship between the amount of mitochondrial protein isolated from normal human skin fibroblasts exactly as described by Janssen et al. (1) and the time course of the NADH oxidase activity in the absence of rotenone. The duration of the first order kinetics decreased after 2–3 min, especially at the highest protein concentrations (Fig. 1). This result indicates that addition of rotenone 4 min after the start of the reaction and measurement of the changes in absorbance during the next 4 min may lead to serious overestimation of the apparent rotenone sensitivity of complex I. To show the consequences of this finding, we performed the assay exactly according to the method described by Janssen et al. (1) by adding rotenone 4 min after the start of the reaction for the protein concentration range shown in Fig. 1. Obviously the complex I activity, if expressed as specific activity (in mU per mg protein), must be unrelated to the protein concentration. We found, however, a statistically significant linear relation between the specific complex I activity and the mitochondrial protein concentration ($R^2 = 0.66, P = 0.004, y = 2.0x + 81.9$, where $y = $ mU/mg protein and $x = $ µg protein/mL), indicating that the complex I assay performed with the method of Janssen et al. is not reliable. Only with highly diluted fractions does the above-mentioned equation approach a constant, but in the regular assay reported by Janssen et al. the protein concentration amounts to 17.6 µg/mL and the reaction is then no longer first order at a reaction time beyond 2 min (Fig. 1).

To find out if the real complex I activity can be assessed when rotenone has enough time to accumulate at the coenzyme $Q_{10}$-binding site of the enzyme complex, we modified the method by measuring the rotenone-insensitive enzyme activity in a separate cuvette, to which we added the inhibitor 1 min before the start of the reaction with NADH. The specific complex

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**Correct Assay of Complex I Activity in Human Skin Fibroblasts by Timely Addition of Rotenone**

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The consequence of this improved procedure is that the required amount of enzyme was doubled, which could be a problem if material is limited. With the use of small cuvettes (5), a concentration of 17.6 μg/mL protein per cuvette volume of 142 μL requires only 2.5 μg, which is equivalent to <1×10^5 fibroblasts.

To establish whether this modification indeed measured the correct amount of enzyme, we used our recently reported method (5) to determine complex I in the mitochondrial-enriched fraction by using coenzyme CoQ10, the water-soluble analog to CoQ10, as the only electron acceptor instead of decylubiquinone plus 2,6-dichloroindophenol. We found that with the use of CoQ10, the assay was less influenced by rotenone-insensitive NADH oxidases, increasing the rotenone-sensitive fraction from mean (SD) of 21% (6%) to 62% (9%) (n = 5; Student 2-tailed t-test, P < 0.005), but there was not a statistically significant difference in the complex I activity of 42.6 (11.4) mU/mg mitochondrial protein compared with that found by the improved method of Janssen et al. A noteworthy observation was that our own method (5) appeared more reliable because the intraassay variation was 18% to 37%, the interassay variation was 27% (n = 5), and the SD of the mean complex I activity was smaller by a factor of 2.

We conclude that for the correct determination of complex I, it is imperative to add rotenone in a separate cuvette before the start of the measurement to allow rotenone to accumulate to its binding site. Otherwise the complex I activity in the mitochondrial-enriched fraction of human skin fibroblasts will be overestimated.

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References


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In Reply

To the Editor:

Of the mitochondrial respiratory chain enzymes, complex I is often regarded as the most difficult to analyze, particularly in tissues with relatively low mitochondrial content, such as fibroblasts. In 2007 we developed a new spectrophotometric assay for complex I of the respiratory chain, in which 2,6-dichloroindophenol (DCIP) is used as terminal electron acceptor for complex I (1). The use of DCIP in a complex I assay has 2 advantages. DCIP helps to keep the quinone used in the assay in the oxidized form, which is desirable because the reduced form (quinol) has been shown to inhibit complex I activity (2, 3). Another advantage is that DCIP has a relatively high molar absorptivity; therefore complex I assays with DCIP produce higher signals than complex I assays that measure NADH oxidation. Using a large panel of fibroblasts and muscle
samples from genetically characterized complex I–deficient patients, we showed that the assay can be applied successfully to diagnose complex I deficiencies. Bénit et al. showed that a similar assay enabled detection of partial complex I deficiency in samples from Harlequin mice (2).

Recently, we started the implementation of the decylubiquinone (DQ)-DCIP based complex I assay on an automated spectrophotometric platform. During the assay implementation, we noticed that blank activity, which is the reaction mixture + rotenone, when measured in a separate cuvette, results in an apparent lower rotenone sensitivity compared to a sequential determination of the blank activity, as in the manual assay described previously (1). This situation is mentioned as a main point in the letter by de Wit et al. We found that measurement of the rotenone sensitivity in a separate cuvette in parallel with the assay cuvette results in a rotenone sensitivity that is around 50% for fibroblast-derived mitochondria (Table 1) and between 70% and 95% for muscle-derived mitochondria (data not shown). These values are similar to recently published observations (2).

To demonstrate that the DCIP-based complex I assay has no intrinsic reproducibility problem, we tested the reproducibility in an automated spectrophotometer. In this way, possible variation due to manual pipetting is eliminated. In addition, we tested 2 coenzyme Q (CoQ) analogs, DQ and CoQ1. The percentage of rotenone inhibition observed with CoQ1 was slightly higher than with DQ, as were the absolute complex I activities measured in isolated mitochondria from fibroblasts (Table 1). The intraassay CV was 4.8% (Table 1). The interassay variation was 7.9% with DQ and 2.5% with CoQ1, compared to 2%–11% for the manual DQ-DCIP method (1), and 41.1% for a recently described CoQ1-based assay (4). The automated assay in which DQ was replaced by CoQ1 gave a further improvement of the %CV (Table 1). From the results presented here we conclude that (1) incubations with rotenone should be performed in parallel with the incubations lacking rotenone, (2) the reproducibility of the DCIP-based assay is below 8%, and (3) CoQ1 appears to give slightly better results than DQ, although this finding should be tested more extensively in a large panel of samples.

On the basis of these findings, we have adapted the protocol for the measurement of complex I in fibroblast-derived mitochondria as follows. A reaction mixture containing 25 mmol/L potassium phosphate buffer (pH 7.6), 3.5 g/L BSA, 0.06 mmol/L DCIP, 70 μmol/L CoQ1 or 70 μmol/L DQ, and 2% (vol/vol) of mitochondrial preparation (1) is preincubated with or without 1 μmol/L of rotenone for 4 min at 37 °C. Subsequently, 0.2 mmol/L NADH is added, and the reaction is followed at 600 nm for 5 min. Complex I activities are calculated essentially as described previously (1). As with the previously published manual protocol, when a nonlinear and rapid DCIP reduction is observed due to an excessively high sample concentration in the reaction mixture, samples are diluted to achieve a linear reaction. The results described in this report were obtained using a Kone 20XTi automated spectrophotometer (Siemens Medical Solutions Diagnostics, Breda, the Netherlands). A full report of the automated complex I assay will be submitted for publication once the validation procedure has been completed.

A final note is that the reduced form of DQ, decylubiquinol, has recently been demonstrated to strongly inhibit complex I activity (2), a phenomenon also observed for other CoQ analogs (2, 3). Therefore we emphasize that the method described here should preferably be used for preparations of isolated mitochondria, because in less pure preparations other cellular NADH dehydrogenases will readily reduce quinines, leading to underestimation of complex I activity.

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Unrecognized Preanalytical Problem with the Spectrophotometric Analysis of Cerebrospinal Fluid for Xanthochromia

To the Editor:

The primary diagnostic test for subarachnoid hemorrhage (SAH) is a computed tomography scan, but because SAH is found in a small percentage of patients with negative computed tomography results, lumbar puncture (LP) and spectrophotometric analysis of cerebrospinal fluid (CSF) for xanthochromia are still needed to make decisions about angiography (1). Oxyhemoglobin (O$_2$Hb) is released from erythrocytes (RBCs) in the CSF in vivo and converted to bilirubin in the leptomeninges in a time-dependent process. Although the trauma of LP frequently produces RBCs in CSF and RBC lysis leads to the presence of O$_2$Hb, no bilirubin is formed in such cases. The presence of bilirubin is considered the most specific sign of SAH, and a LP is recommended 12 h after the appearance of symptoms to test for the presence of bilirubin (2,3). O$_2$Hb and bilirubin have absorbance peaks at 413–415 nm and 450–460 nm, respectively, but because of the overlap in absorbance wavelengths with O$_2$Hb, bilirubin is preferentially measured at 476 nm (2–4). Nevertheless, avoidance of hemolysis ex vivo will improve the analysis of bilirubin in CSF and may make O$_2$Hb a more reliable marker of bleeding. We conducted experiments to elucidate why RBCs lyse in CSF.

A CSF pool was prepared from nonpathologic CSF samples and stored at $-80\, ^\circ\!\!\text{C}$ (approval from regional ethics committee not required). After thawing, RBCs that had been obtained from volunteers with informed consent were added to the CSF pool and a physiological saline solution to final RBC concentrations of 20–100 $\times 10^9$/L. We then centrifuged vials with the solutions at 2000g for 5 min immediately and at hourly intervals up to 4 h after RBC addition and measured hemolysis by monitoring the absorbance at 415 nm (Thermo Spectronic Unicam UV300). Fig. 1 shows the considerable RBC- and time-dependent hemolysis values we obtained. In physiological saline, only slight hemolysis was observed, even after 4 h (maximum, 0.039 absorbance units; data not shown). Interestingly, the pH of the CSF

References

2. Bénit P, Slama A, Rustin P. Decylubiquinol imidazole redox titrations played no role in the design of the study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Fig. 1. Hemolysis of RBCs added to a pool of CSF during a 4-h incubation at ambient temperature.

Hemolysis was measured by monitoring the supernatant absorbance at 415 nm. The 3 upper curves are results for the original CSF pool (pH 8.8), and the 3 lower curves are results obtained after preincubating the CSF pool in a 5% CO$_2$ atmosphere, producing an approximate pH of 7.4. RBCs were added to final concentrations of $20 \times 10^9$/L (●), $50 \times 10^9$/L (■), and $100 \times 10^9$/L (▲) in the CSF pool at pH 8.8, and to final concentrations of $20 \times 10^9$/L (○), $50 \times 10^9$/L (□), and $100 \times 10^9$/L (△) in the CSF pool at pH 7.4.


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1 Nonstandard abbreviations: SAH, subarachnoid hemorrhage; LP, lumbar puncture; CSF, cerebrospinal fluid; O$_2$Hb, oxyhemoglobin; RBC, erythrocyte (red blood cell).
pool was 8.8 (Radiometer ABL725), and HCO₃⁻ and the pCO₂ were undetectable. Thus, the pH increase was likely caused by the evaporation of CO₂ during handling and storage. After we subsequently incubated the CSF pool in a CO₂ chamber containing 5% CO₂, the pH decreased to approximately 7.4, reflecting physiological pH. When we repeated the experiment described above at this pH, we found almost no hemolysis (Fig. 1), even at the highest RBC concentration.

We then obtained 53 consecutive routine CSF samples from neurorosurgical patients suspected of SAH and analyzed the samples within 1–2 h of collection. The mean (SD) pH was 8.07 (0.12). Hemolysis is also considerable in CSF at pH 8.0, approximately 20%–40% of the hemolysis observed at pH 8.8 during the first hour, although the increase is less at subsequent hours. Thus, routine samples obtained for spectrophotometric analysis most often have a pH at which preanalytical hemolysis takes place.

When 1 mL CSF (equilibrated to pH 7.4) was added to 6- to 10-mL vials usually used for LP, the pH increased at a rate of 0.01 pH units/min during the first hour (slightly faster during the first 30 min), which is equivalent to an increase in the pH to 7.8–8.0 after 1 h. Agitation of the vial speeds up this process. The pH changes were much less in a 2-mL vial containing 1 mL CSF, and the physiological pH is conserved if the vial is completely filled.

The results show that the amount of hemolysis depends at least partly on the pH of the CSF sample and that the pH of CSF increases ex vivo upon exposure to atmospheric air owing to the evaporation of CO₂. If the volume of air above the CSF in the vial is small and the vial is capped quickly, changes in pH are reduced substantially, with almost negligible hemolysis ex vivo. Surprisingly, no publications have described this preanalytical problem, although several have shown that the presence of RBCs after a traumatic tap may cause a falsely increased O₂Hb in the CSF sample (3, 5).

Evaporation of CO₂ is well known to lead to a pH increase in fluids containing HCO₃⁻. The nonbicarbonate buffering capacity in CSF is low because of the low protein concentration, and the pH increases quickly if the sample is exposed to air. The use of a small vial completely filled with CSF and capping the vial immediately after the LP will minimize the problem and improve the analysis, because the presence of O₂Hb may affect the ability to accurately detect bilirubin. This simple step may even improve the reliability of detecting O₂Hb as a marker of SAH sooner than the prescribed 12 h, but the impact of this approach needs to be shown in a clinical setting.

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Diagnosis of Metachromatic Leukodystrophy by Immune Quantification of Arylsulphatase A Protein and Activity in Dried Blood Spots

To the Editor:

Metachromatic leukodystrophy (MLD),¹ an autosomal recessive neurodegenerative disease resulting from a deficiency of arylsulfatase A (ASA), results in the lysosomal accumulation of sulfatide in several peripheral organs, notably the central nervous system. The

¹ Nonstandard abbreviations: MLD, metachromatic leukodystrophy; ASA, arylsulfatase A; ASA-PD, ASA pseudo-deficiency; DBS, dried blood spots.
carrier frequency of MLD at 1:152, this frequency will lead to a prevalence of compound heterozygotes as high as 1:2300. Diagnostic laboratories are often required to perform additional complex sulfatide assays or molecular analysis to obtain a definitive diagnosis.

We have developed and evaluated 2 immune-based assays that enable the differentiation of MLD individuals from individuals with ASA-PD and unaffected controls. These assays use an immune-quantification assay to quantify the amount of ASA protein and an immune-capture activity assay to determine enzyme activity. Both assays are performed on 3-mm dried blood spots (DBS) collected on filter paper.

Affinity-purified sheep anti-ASA polyclonal antibody was produced and Eu$^{3+}$-labeled as previously described (3). Recombinant human ASA protein was expressed in a CHO-K1 expression system from a full-length ASA cDNA clone in HT 14/CP 8 pBluescript® vector. Calibrators and quality control materials were prepared by diluting recombinant human ASA in working buffer (0.1 mol/L sodium acetate/acetic acid, 0.1% heat-treated BSA, pH 5.0) or Delfia® assay buffer (Perkin-Elmer Life Sciences) to measure ASA activity and protein, respectively.

All DBS were stored at −20 °C before analysis. The institute’s ethics committee approved use of the patient samples. ASA protein from DBS was assayed using a single 3-mm disk as described previously (3). A calibration curve (2.0–1000 pg/well) was included in each assay. ASA activity in DBS was determined as described previously (4), using two 3-mm disks per assay, 0.1 mol/L sodium acetate/acetic acid, 0.1% heat-treated BSA, pH 5.0, as elution buffer; 20 mmol/L sodium acetate/acetic acid, pH 5.0, as wash buffer; and 5 mmol/L 4-methylumbelliferyl sulfate in 0.2 mol/L sodium acetate/acetic acid, pH 5.0, 0.1% BSA (100 μL/well) as substrate. A calibration curve using recombinant human ASA (2.0 –800 pmol/h/well) was included in each assay.

The ASA protein and activity assays gave linear responses over the biological range ($R^2 > 0.999$). The detection limits were 0.8 pg/well and 1.5 pmol/h/well. The intra- and interassay CVs were 9% and 10%, respectively, for the protein assay and 6% and 13% for the activity assay. The median amount of ASA protein in whole blood from unaffected, ASA-PD, and heterozygote individuals was 31.9 μg/L (range 21.0–46.3 μg/L), 13.0 μg/L (range 8.3–17.0 μg/L), and 12.5 μg/L (range 12.0–13.0 μg/L), respectively; no detectable ASA protein was observed in MLD individuals (Fig. 1A). The median ASA activity in unaffected and heterozygote individuals was 4.16 μmol/min/L (range = 1.48–7.72 μmol/min/L) and 2.62 μmol/min/L (range = 2.44–2.80 μmol/min/L); no detectable activity was observed in ASA-PD or MLD individuals (Fig. 1B).

ASA activity did not enable differentiation of ASA-PD individuals from MLD patients; however, the ASA protein quantification assay enabled clear differentiation between controls and ASA-PD and MLD individuals. To further investigate why ASA-PD showed no ASA activity, we analyzed DBS from an unaffected individual. This DBS was stored at room temperature for up to 30 days, and during this period ASA activity decreased such that only 39% of activity remained after 30 days; in contrast, no decrease was observed in the ASA protein. Measured ASA protein and activity in heterozygotes were both in the lower end of the reference interval.
The ability to use DBS to measure ASA protein and activity will simplify procedures for collection, handling, and storage of patient samples. The ease of transporting DBS, combined with the diagnostic sensitivity and specificity of these assays, provides a powerful approach to the diagnosis of MLD. After further investigation and validation, these assays may be used for newborn screening for MLD and other lysosomal storage disorders. Importantly, the inability to differentiate ASA-PD from ASA by the activity assay alone suggests that ASA protein determination from DBS may be the most appropriate screening approach for newborns, as has recently been proposed in a report of a multiplex immune-quantification assay (5).

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Circulating Matrix Metalloproteinase-7: An Early or Metastatic Marker for Renal Cell Carcinoma?

To the Editor:

We read with great interest the article by Sarkissian et al. (1) reporting the identification of serum matrix metalloproteinase-7 (MMP-7)1 as a marker for renal cell carcinoma (RCC). For the purpose of identification, the authors used sera from healthy individuals and RCC patients and combined 2-dimensional electrophoresis of an RCC cell-line supernatant and an immunoblotting procedure. Subsequently, the authors measured serum MMP-7 concentrations with a self-developed immunoassay. Higher concentrations were found in RCC patients than in healthy individuals (7.56 vs 2.13 μg/L), whereas serum MMP-7 concentrations in patients with localized RCC did not differ from concentrations in patients with metastatic RCC (7.26 vs 7.87 μg/L). These results suggest that MMP-7 could be a useful marker for detecting RCC during the early stages of the disease. These findings are of utmost importance because at present no blood marker is known for the early detection of RCC.

Continuing our studies on the diagnostic performance of MMPs, we investigated circulating MMP-7 in RCC patients, but our results (2) differed from those of Sarkissian et al. We measured MMP-7 in heparin-plasma samples using Fluorokine multianalyte profiling assays (R&D Systems). In our study MMP-7 concentrations (Fig. 1) in healthy controls were not found to be different from those in RCC patients with localized cancer.

References

1. Nonstandard abbreviations: MMP-7, matrix metalloproteinase-7; RCC, renal cell carcinoma.
Letters to the Editor

Fig. 1. Scatterplots of MMP-7 concentrations in plasma of healthy controls and patients with renal cell carcinoma.

Short horizontal lines with respective figures indicate the medians of the groups and the dotted horizontal line represents the cutoff point of 3.35 \( \mu \text{g/L} \) calculated as the 95th percentile of the control group by use of the nonparametric approach for establishing reference limits. We found significant differences between the study groups by using the Kruskal–Wallis test \((P < 0.0001)\) with Dunn post hoc comparisons indicated by the following symbols: a, compared to controls; b, compared to the N0M0 group (with localized cancer); c, compared to the N1M0 group (with lymph-node involvement); and d, compared to the M1 group (with distant metastases) \((all \ P < 0.05)\). Adapted from (2).

(N0M0 stage; TNM-1997 classification) \((1.64 \mu \text{g/L}, n = 50 \text{ vs } 1.85 \mu \text{g/L, } n = 39)\), but were markedly higher in patients with lymph-node involvement \((N1M0 \text{ stage, } n = 13; 3.82 \mu \text{g/L})\) or distant metastases \((M1 \text{ stage, } n = 45; 3.34 \mu \text{g/L})\). These data do not support the use of circulating MMP-7 as a marker for detecting early stages of RCC.

The clinical importance of these data makes it worthwhile to consider possible reasons for the disparities between these 2 studies regarding the clinical validity of serum MMP-7 measurements. Preanalytical and analytical factors as well as aspects of study design should be considered. One reason may be differences in blood sampling, which is well known to be a significant preanalytical determinant of MMP measurements (3). Sarkissian et al. (1) used sera for their measurements, whereas we used plasma. Circulating MMP-7 values, however, are not greatly affected by the blood collection procedure (3); therefore blood sampling seems an unlikely explanation for the discrepant findings. Analytical variables seem more likely to be causes of the discrepant findings. The 2 studies used different antibodies in the MMP-7 assays. In consequence, different epitopes of MMP-7 may have been recognized, leading to differing detection of the various forms of MMP-7. MMP-7 exists in human serum as polymers or complexed with serum proteins (4). According to the information from the manufacturer, the assay that we used measures pro- and active forms of MMP-7 as well as MMP-7 complexed with tissue inhibitor of MMP 1. The assay used by Sarkissian et al. (1) detected only pro- and active MMP-7, but the detection of MMP-7 forms complexed with serum proteins cannot be excluded. Sarkissian et al. observed higher MMP-7 concentrations in their patients (1) than we did in ours, and more importantly, they found increased MMP-7 concentrations in both the patients with localized cancer and the patients with metastases. In contrast, the 95th percentile they observed in their control group \((3.29 \mu \text{g/L vs our 3.35 } \mu \text{g/L})\) suggested that these concentration differences did not exist in the healthy participants of the 2 studies. Because the same recombinant human MMP-7 was used as a calibrator in both assays, it can be assumed that MMP-7 forms possibly typical for RCC patients are more sensitively detected by the assay of Sarkissian et al. (1) than by the R&D MMP-7 assay we used. Different assay designs including the use of the mentioned specific antibodies could affect the assay sensitivity, but autoantibodies to pro–MMP-7 occurring in sera of RCC patients (1) could also interfere with measurement of MMP-7. The differences in study results may also be attributable to different approaches used for selection of study participants and the sizes of study groups. The study of Sarkissian et al. (1) was designed to identify proteins related to RCC rather than to validate the clinical utility of the analyte. In contrast, our study focused on the potential diagnostic and prognostic performance of MMP-7 in somewhat larger and well-classified RCC groups. Sarkissian et al. (1) may have been unable to demonstrate an association of MMP-7 with advanced RCC owing to the limited number of RCC patients in their study population. Finally, MMP-7, although predominantly expressed by tumor cells, may also be released from other cell types (2, 5). For this reason, various clinical conditions may influence the concentrations of circulating MMP-7 in study populations and contribute to such variation in data. Prospective studies will be required to clarify the clinical applicability of circulating MMP-7 as an early or metastatic marker in RCC patients.
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References


Strongly Increased International Normalized Ratio with Recombinant Neopterin R* Compared with Tissue Extract Neopterin Plus® in Patients Initiating Oral Anticoagulant Therapy: Implications for Anticoagulation Dosage

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Strongly Increased International Normalized Ratio with Recombinant Neopterin R* Compared with Tissue Extract Neopterin Plus® in Patients Initiating Oral Anticoagulant Therapy: Implications for Anticoagulation Dosage

To the Editor:

High-accuracy determination of the international normalized ratio (INR)1 is of importance in controlling treatment of patients with oral anticoagulants. The INR is defined according to a calibration model adopted by the WHO (1) as the normalized prothrombin clotting time (PT/MNPT)ISI, where MNPT is mean normal prothrombin time of 20 healthy individuals. The factor ISI (international sensitivity index) compensates for the differing sensitivities of thromboplastin reagents used to measure prothrombin clotting time. Thromboplastin reagents can vary widely in their sensitivities to clotting factor deficits (induced by coumarin treatment), resulting in INR differences (2). Recombinant thromboplastins have been discussed as being more sensitive to variation in coagulation factor VII (FVII) than tissue extract thromboplastins (2–4). FVII has a relatively short plasma half-life of 6 h compared to the half-lives of factor X (45 h) and prothrombin (60 h). The increased sensitivity for FVII may be observed during unstable anticoagulation, such as in the initial phase of oral anticoagulant therapy, and may lead to differences in observed INRs that have implications for subsequent oral anticoagulant dosing advice.

To investigate this phenomenon, we compared the novel recombinant Neopterin R PT reagent (ISI 1.08; Roche Diagnostics GmbH) with the tissue extract PT reagent Neopterin Plus (ISI 1.29; Roche Diagnostics GmbH) in both stable oral anticoagulant patients and patients recently started with acenocoumarol. We collected citrate samples from 25 patients starting with a standard loading dose of acenocoumarol (day 1, 6 mg; day 2, 4 mg; day 3, 2 mg) between 3 and 7 days after initiating oral anticoagulation and from 20 patients on stable acenocoumarol therapy (therapeutic INR range 2.0–4.0). We measured PT using a STA-R analyzer (Roche Diagnostics GmbH).

We found excellent linear correlation (r² = 0.94; slope 1.31; intercept −0.51) between recombinant Neopterin R and tissue extract Neopterin Plus for patients on stable oral anticoagulant therapy. The INR differences are plot-
We conclude that recombinant Neoplastin R shows linear correlation with tissue-extract Neoplastin Plus for patients on stable oral anticoagulation with acenocoumarol. For patients in the initial phase of oral anticoagulation, we also found a linear correlation, although with increased slope. Above the therapeutic range, we observed values that were almost 2-fold increased for patients in the initial phase of oral anticoagulant therapy with recombinant Neoplastin R. It is known that the INR does not give consistent results across thromboplastins during the induction phase of oral anticoagulation, because the WHO model requires plasma from stabilized patients. However, protocols for anticoagulation treatment require INR measurement after a coumarin loading dose to define subsequent doses, and it has been reported that the INR scale can be used to control anticoagulation even in the early phase. Therefore, it is very likely that the observed sensitivity of recombinant Neoplastin R in patients initiating oral anticoagulation will influence subsequent anticoagulation dosing. Lower coumarin doses would be prescribed because of the increased INRs with recombinant Neoplastin R vs tissue-extract thromboplastin and could delay the time necessary to reach stable anticoagulation. In consequence, patients might experience prolonged anticoagulation with heparin, increased bleeding, or thrombotic complications. Our findings may also have implications, such as adaptation of dosing algorithms, for other recombinant thromboplastins used in the management of oral anticoagulant therapy.

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**References**


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Serum 25-OH Vitamin D2 and D3 are Stable under Exaggerated Conditions

To the Editor:

Vitamin D analysis is increasingly performed with HPLC–tandem mass spectrometry instead of RIA, and hence samples are frequently sent to more specialized centers for analysis. We therefore investigated the stability of both 25-OH vitamin D3 (25-OH D3) and 25-OH vitamin D2 (25-OH D2) in serum stored under extreme conditions that would likely exceed those normally encountered in sample transit and storage.

After our study received ethics committee approval, we collected 200 mL of blood from a single donor who had given written informed consent and had been previously been determined to have adequate concentrations of 25-OH D3. After centrifugation of the blood sample the serum was removed and supplemented with 50 nmol/L of authentic 25-OH D2 to boost endogenous concentrations. We then transferred 1.0-mL portions into 5-mL clear polystyrene tubes. The sample-containing tubes were capped, stored at −20 °C, and subsequently subjected to various treatments in replicates of 5. These treatments included multiple freeze-thaw cycles (1 to 5 cycles), 8 days at ambient temperature under various conditions, and brief exposure to artificial ultraviolet light. The ambient-temperature storage conditions were as follows: 1 set of 5 samples was left on the laboratory bench uncovered and exposed to fluorescent light and diffuse sunlight for 8 days at 20 °C;

Fig. 1. Box-and-whisker plots of measured concentrations of plasma 25-OH D3 (filled boxes) and total plasma 25-OH-vitamin D (D2 + D3) (open boxes) after samples were subjected to 11 various treatments.

Treatments were 1–5 freeze-thaw cycles; 8 days left at ambient temperature in the laboratory with diffuse sunlight (8 days ind diffuse); 8 days ambient temperature indoors exposed to direct sunlight (8 days ind direct sun); 8 days ambient temperature outdoors exposed to direct sunlight (8 days out direct sun); 4 × 30-min exposure to artificial ultraviolet light (2h UV light); 8 days ambient temperature indoors but covered and protected from direct sunlight (8 days ind covered); and 8 days ambient temperature outdoors but covered and protected from direct sunlight (8 days out covered). Boxes represent the lower and upper quartiles with medians and the whiskers represent the 10th and 90th percentiles. Treatments showing significant differences with pair-wise multiple comparison are denoted for plasma 25-OH D3 (\(P = 0.012\)) and total plasma 25-OH-vitamin D (D2 + D3) (\(*P < 0.001\)) using pair-wise multiple comparison.
2 sets of 5 samples were left indoors, inside the author’s solarium, with 1 set uncovered and exposed to direct sunlight and the second set covered and protected from sunlight, at temperatures ranging between 30 °C in the day and 5 °C at night; and 2 sample sets of 5 were left outdoors (outside the building), with 1 set uncovered and exposed to direct sunlight and the other set covered and protected from sunlight, with similar temperature ranges. Sample exposure to artificial ultraviolet light was for 4 periods of 30 min, 30 cm from an ultraviolet lamp used for sterilization within a biohazard hood.

Vitamin D was analyzed after hexane extraction and reconstitution in 70% aqueous methanol by HPLC–tandem mass spectrometry. We used a Shimadzu 20 series HPLC system with a C8 reversed-phase column coupled to an API 3200 Q-trap tandem mass spectrometer under conditions similar to those described previously (1). The calibrator range for 25-OH D2 and 25-OH D3 was 5–320 nmol/L. The internal standard was [2H₆]-25-OH D3 (20 ng), and the positive ion transitions were m/z 413.3/395.4 for 25-OH D2, m/z 401.3/383.4 for 25-OH D3, and m/z 407.3/389.4 for [2H₆]-25-OH D3. All samples were analyzed in the same assay, and the within-run CV for the QC material was 10.2%. Results for 25-OH D3 and the total 25-OH-vitamin D (D2 + D3) are shown in Fig. 1. One-way repeated measures of ANOVA were used to determine whether any treated sample group was significantly different from the others. Uncovered samples at ambient temperatures that had been subjected to 8 days of treatment either inside or outside with exposure to direct sunlight and variable temperature were found to have significantly lower amounts of both 25-OH D3 (P = 0.012) and 25-OH D2 (P = 0.002) compared to all other treated samples, which were themselves not significantly different. Covered samples at ambient temperatures that were stored either inside or outside were unaffected, indicating that it was the prolonged exposure to direct sunlight that affected both 25-OH D3 and 25-OH D2 concentrations in the samples.

In a study in which samples were exposed to up to 11 freeze-thaw cycles, no detrimental effects were observed for 25-OH D3 and 25-OH D2 measured with a binding assay (2), although these conditions may affect the specificity of the results. Similarly, concentrations of 25-OH vitamin D, measured with 2 different immunoassays, were found to be unaffected in samples exposed to multiple freeze-thaw cycles (3, 4). Here we addressed the question of 25-OH D3 and 25-OH D2 stability determined by mass spectrometry. We found that under extreme conditions both 25-OH D3 and 25-OH D2 are exceptionally stable, except when subjected to prolonged unprotected exposure to direct sunlight. However, serum samples for 25-OH D3 and 25-OH D2 analysis would normally be shipped inside containers that would protect them from direct sunlight and therefore would require no special transport or storage considerations other than those required by regulatory agencies. Our findings should be useful to laboratories that send samples to specialized centers for 25-OH D3 and 25-OH D2 analyses.

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